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THE PROPAGATION OF THE FUJINAMI FOWL-MYXOSARCOMA IN ADULT DUCKS.

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FUJINAMI and Hatano (1929) discovered that a certain myxosarcoma of the fowl can be propagated in a second species of bird, the duck. My colleague, Dr. W. E. Gye (1931, 1932), in the course of a study of the properties of the Fujinami tumour, has been able to confirm this discovery; he, too, has been able to grow and propagate the tumour in ducks. Gye, however, met with difficulties. He found that the conditions under which the tumour will grow freely in ducks are restricted. He found, for example, that the tumour will not grow as readily in some varieties of ducks as in others. The Khaki Campbell variety proved the most sensitive of the few he tested, and practically all his observations have been made on ducks of this kind.

Gye found that in Khaki Campbell ducks the clinical course of the disease differs according to the age of the host. When he used very young ducklings they almost always died of the disease—in about nine days as a rule; when he used ducklings which were older than about one month he still obtained tumours regularly, but the tumours never grew to large size, never killed the host, and always retrogressed early. In adult ducks of this same variety Gye found that the results of his inoculations were much the same as in month-old ducklings, and it mattered little whether tumour filtrates or tumour-cells were used as the inoculum.

In his attempts to transfer the Fujinami tumour from duck to duck through an indefinite series of tumour generations Gye only succeeded when he used young ducklings. But it is to be remarked that he did not test the effect of unusually large doses of minced tissue. Throughout his work he used only such doses as are commonly used in this country, and regarded as entirely adequate, even optimal, for the propagation of tumours by means of cell-grafts. Thus Gye's doses were of the order of 0·05 c.c. of tumour mince. The doses used by Fujinami and his colleagues are unknown to us.

It has now been found, as will be recorded below, that when minced Fujinami tumour is injected into adult ducks the amount injected materially affects the results observed. For example, on January 30th, 1932, two adult Khaki Campbell ducks each received, both into the right leg and into the left breast, 0·5 c.c. of tissue mince prepared from a Fujinami tumour which had been grown in a fowl. Nine days later one of these ducks was dead; a large soft tumour had developed at the site of each of the two injections. The second duck did not die. A large tumour developed at the site of each injection, just as in the first duck, and the bird became ill and lame. For several weeks the condition of this second bird remained practically unchanged, and then its tumours began to decrease very slowly in size. Eventually the tumours disappeared completely, and the duck regained its normal condition except for a slight but permanent limp.

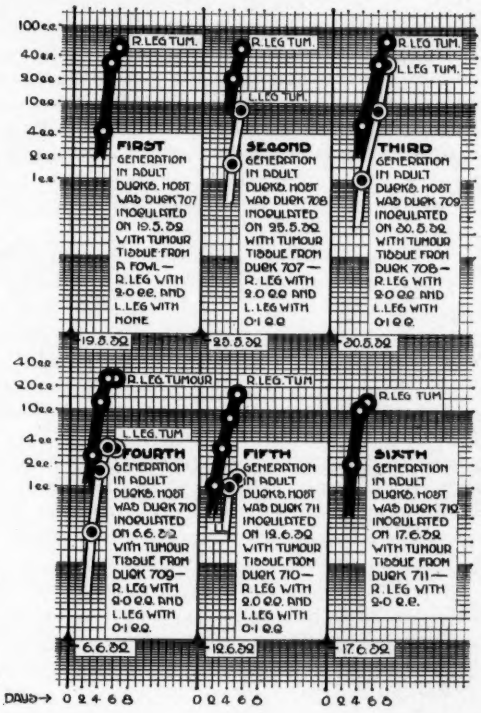
This greater efficacy of unusually large doses of cells has been confirmed in many experiments upon adult ducks. Usually the ducks have been injected in one site only—a leg—and usually the dose injected has been 2·0 c.c. of bubble-free mince. By the fourth or fifth day following the injection large tumours have always developed, but the subsequent history of the tumours has not been uniform. Often the tumours have continued to grow at an undiminished rate, and have killed the host within a few days; at other times the rate of growth of the established tumour has become steadily slower and slower, has finally ceased, and then has given place to slow or rapid regressive changes, which have ended with the disappearance of the tumours and the complete recovery of the hosts. Sometimes an intermediate condition has been observed; here, although the rate of growth of the tumour has definitely begun to fall, the host has eventually succumbed.

Since the injection of large doses of minced fowl-grown tissue thus gives rise to large, rapidly-growing tumours in adult ducks, ample quantities of duck-grown tissue of high activity are readily available for investigation. One experiment which has been carried out is summarized in the chart which constitutes the accompanying figure. The primary object of this experiment was to find whether, when large doses of minced tissue are used, it becomes possible to transfer the Fujinami myxosarcoma from one adult Khaki Campbell duck to another through an indefinite number of tumour generations. Gye had been able to propagate the tumour only in young ducklings, and it seemed desirable, as a further object of the experiment, to ask why he was thus limited. In particular it seemed worth while to inquire whether Gye's difficulties arose from some qualitative difference between the tumours growing in young ducklings and those growing in adults. These secondary considerations somewhat influenced the form of the experiment. In testing whether older ducks can be used for the propagation of the Fujinami tumour the experiment was not without an immediately practical bearing. Hitherto the maintenance of a strain of Fujinami myxosarcoma in Khaki Campbell ducks has been possible only when a continuous succession of young ducklings has been provided; the provision of such a succession is both laborious and costly. It is proposed to describe and discuss the experiment summarized in the chart at some length.

The ducks used in the experiment were all healthy young adults of the

Khaki Campbell variety. All had been hatched at the end of March, 1932. At the beginning of the experiment their weights ranged from 1440 to 1860 gm.

The method of preparing the mince differed from that commonly used in this laboratory. Ordinarily tumour tissue is clipped finely with scissors—often after it has first passed through a Denne mincer—and is injected without further treatment. But such mince generally contains air in the form of tiny bubbles; sometimes, indeed, the bubbles are so numerous that the mince can


100 c.c.
40 c.c.
20 c.c.
10 c.c.
4 c.c.
2 c.c.
1 c.c.
R. LEG TUM.
L. LEG TUM.
FIRST GENERATION IN ADULT DUCKS. HOST WAB DUCK 707 INOCULATED ON 19.5.32 WITH TUMOUR TISSUE FROM A FOWL — R. LEG WITH 2·0 c.c. AND L. LEG WITH NONE.
SECOND GENERATION IN ADULT DUCKS. HOST WAB DUCK 708 INOCULATED ON 25.5.32 WITH TUMOUR TISSUE FROM DUCK 707 — R. LEG WITH 2·0 c.c. AND L. LEG WITH 0·1 c.c.
THIRD GENERATION IN ADULT DUCKS. HOST WAB DUCK 709 INOCULATED ON 30.5.32 WITH TUMOUR TISSUE FROM DUCK 708 — R. LEG WITH 2·0 c.c. AND L. LEG WITH 0·1 c.c.
19.5.32
25.5.32
30.5.32
R. LEG TUMOUR
FOURTH GENERATION IN ADULT DUCKS. HOST WAB DUCK 710 INOCULATED ON 6.6.32 WITH TUMOUR TISSUE FROM DUCK 709 — R. LEG WITH 2·0 c.c. AND L. LEG WITH 0·1 c.c.
FIFTH GENERATION IN ADULT DUCKS. HOST WAB DUCK 711 INOCULATED ON 12.6.32 WITH TUMOUR TISSUE FROM DUCK 710 — R. LEG WITH 2·0 c.c. AND L. LEG WITH 0·1 c.c.
SIXTH GENERATION IN ADULT DUCKS. HOST WAB DUCK 712 INOCULATED ON 17.6.32 WITH TUMOUR TISSUE FROM DUCK 711 — R. LEG WITH 2·0 c.c.
6.6.32
12.6.32
17.6.32
DAYS → 0 2 4 6 8
0 2 4 6 0
0 2 4 6 0

be accurately described as a froth containing tissue particles. This frothing is unavoidable; it is due to the peculiar tenacity of the tissue fluids of the Fujinami tumour, and the churning action of the scissors. These bubbles of air were removed, by means of a vacuum pump, from all mince used in the experiment under discussion. Consequently the mince used in the experiment was both more accurately measurable and more highly concentrated—more tissue and tissue fluids per c.c.—than the mince ordinarily used in this laboratory.

The method by which the tumour sizes were obtained and the curves of growth plotted may perhaps receive mention, although these have been described and discussed already (Gye and Purdy, 1931). The sizes were

estimated, both during life and after death, by comparing the actual tumours with dummy tumours of known volumes. From the data thus obtained curves were plotted on semi-logarithmic paper. Such curves permit ready comparison of *rates* of growth. A given rate is always represented by a line which has the same inclination whatever the size of the tumour. Constant rate of growth is indicated by a straight line. Any change in the inclination of the curve indicates a change in rate of growth, and the more nearly horizontal the direction of the curve the slower will be the rate of growth which is indicated.

Another point to which attention may be drawn is the effort made in this experiment to transplant early. Sometimes the tissue for inoculation into the new host was taken immediately upon the first sign of slackening in the rate of growth of the tumour ; sometimes it was taken earlier ; it was never taken later. It seemed reasonable to suppose that tumour-tissue taken before retrogressive changes had become far advanced would be more certainly active than tissue taken at a later period.

The main results of the experiment can be gathered from the chart. Starting with duck 707, which received mince made from fowl-grown Fujinami tissue, the tumour was propagated in young adult ducks for six generations, the total time occupied being 34 days. The tumour-tissue of the sixth generation in ducks appeared, post-mortem, to be as soft and healthy as that of any of the previous generations had been, and at the end of the experiment there seemed every reason to suppose that, by the technique adopted for the experiment, the Fujinami myxosarcoma can be propagated in adult Khaki Campbell ducks with ease through an indefinite number of tumour generations. This, then, was the answer to the primary question posed in the experiment : the tumour can be propagated in adult ducks.

The secondary object of the experiment took the form of an investigation of the growth energies possessed by different tumours. It seemed conceivable, for instance, that a tumour which arises from a large inoculum might possess greater growth energy than one which arises from a small inoculum. That in adult ducks there is no such difference is shown, however, by the growth-curves of ducks 708, 709 and 710. Each of these ducks received 2·0 c.c. of tumour mince into the right leg, and 0·1 c.c. into the left. In each duck, as was expected, the tumour developing from the smaller dose appeared rather later than the one developing from the larger, but thenceforward the rate of growth —and presumably also the rate of multiplication of the malignant cells themselves—was the same in both tumours. The similarity between the two tumours of each pair is shown by the fact that the two growth-curves run parallel.

From these observations it is concluded that in adult Khaki Campbell ducks the greater efficacy of large doses of minced Fujinami tumour is due simply to the circumstance that the tumour is started at a larger number of primary foci, and on that account reaches larger size before regressive changes have time to appear.

Again, since the tumour arising from a small dose of mince kills a young duckling with certainty, whilst the tumour arising from a similar dose given to an adult is in no way dangerous to life, it seemed conceivable that there might be some corresponding difference in the growth energies of the tumours.

Careful observations are wanting. My own experience suggests that over the corresponding period, the tumour in an adult duck grows just as quickly as the tumour in a duckling; that, for instance, the tumour arising from 0·1 c.c. of mince which has been injected into the leg of a duckling would give just such a curve as was given by the small tumours in ducks 708, 709 and 710. It is clear that there can be no great difference, and that the malignant cells of daughter-tumours start out with like growth energy whether in young or in old ducks—provided, of course, that the same sample of tumour-mince has been used. Thus the *age* of the tissues of the host can play no great part in determining the inherent energy of growth possessed by the malignant cells of a tumour developing from inoculated mince.

From these considerations it is clear that the hitherto puzzling differences in clinical course which tumours run in ducks of different ages are chiefly or wholly dependent upon the relation which the dose of mince bears to the size of bird. Relative to the *size* of the duckling, the doses of tissue-mince used by Gye were enormous, and so were the tumours produced before death intervened, which was at about the ninth day—approximately the time at which adult ducks die when overwhelmed by tumours developing from 2·0 c.c. doses of minced tissue.

It is found that the form of growth of a tumour is influenced by the size of bird in which it is growing, rather than by the absolute size of the tumour. Thus a tumour which is huge in comparison with the limb in which it is growing is more compact and has a relatively small admixture of normal tissue. From this circumstance it follows that a proportionally larger amount of tissue can be collected from a tumour produced by an inoculum which is large in comparison with the size of the bird.

So far as can be seen, then, the difficulties which Gye met in his attempts to propagate the Fujinami myxosarcoma in Khaki Campbell ducks were not due to qualitative differences in the tumours he produced. That he was successful only when he used young ducklings must be attributed chiefly or solely to his choice of dose. When allowance is made for difference in concentration, his doses of mince appear to have been of the order of 0·025 c.c. as compared with the 2·0 c.c. used for the series of ducks described above. Probably if Gye had taken his tissue before severe regressive changes had had time to develop, and if he had found it possible to work with a relatively small yield of readily available tumour-tissue, he, too, in spite of his small doses, might have been able to propagate the Fujinami tumour in adult Khaki Campbell ducks. The direct test has not been made, but the advantage of the large inocula now seems to be largely mechanical.

Another characteristic of the Fujinami tumour growing in adult ducks is illustrated, though imperfectly, in the growth curves shown in the chart. This is the tendency to regression. Gye (1931, 1932) has already drawn attention to this puzzling phenomenon. It occurs no matter whether the tumour has arisen from mince prepared from tumours grown in fowls or in ducks—indeed the whole clinical course of a tumour seems to be unaffected by the species from which the inoculum has come. That these regressive changes are not due directly to the mere mass of the tumour—as, for instance, to a progressive failing of the blood-supply as the tumour grows—is now known

from the fact that a 2·0 c.c. dose of mince will often give rise to a huge tumour, which kills an adult duck before regressive changes appear. It is shown, too, by the tumours of duck 710, which, though widely different in size, both stopped growing at exactly the same time. In fact, it is even unknown as yet whether regression is brought about primarily by changes originating in the host or by changes originating in the cell itself. The history of the tumours of duck 710, for instance, gives no indication. Both tumours began to regress at exactly the same time, but both alike had been exposed to the same influences originating in the host, and in both the tumour-cells had passed through the same number of tumour generations.

Whether the age of the host affects the time at which the regressive changes appear has not been investigated. The doses of mince given to small ducklings have always been too large. The resulting tumours have always killed before the time at which regressive changes might be expected to appear.

One further remark in reference to the experiment illustrated in the chart: It is perhaps advisable to point out that the maintenance of the tumour malignancy in this experiment may have been fortuitous. Only one duck was used at each transfer of the tumour. It would be unsafe, however, to assume from this experience alone that the Fujinami tumour will always maintain a highly malignant form when only one duck is used at each passage. The series of passages was much too short to permit of such a deduction. When a continuous series of highly malignant tumours is desired it would seem wise—at least until further experience has been gained—to use several ducks at each transfer and to transfer only from the most rapidly growing tumour of each generation. This plan is usually found advantageous in the propagation of tumours.

SUMMARY AND CONCLUSIONS.

Fujinami and Hatano found that a certain myxosarcoma of the fowl is propagable in ducks.

Gye was able to confirm the observation in ducks of the Khaki Campbell variety, but only by using very young ducklings.

It is now found that by the use of doses of tissue much larger than those given by Gye, it is possible to propagate the Fujinami myxosarcoma in adult ducks of the Khaki Campbell variety through an indefinite number of generations.

The greater efficacy of a large dose of tumour-mince is not due to any greater energy of growth conferred upon the cells of the resulting tumour. It is due to the fact that the resulting tumour starts in a greater number of primary foci, and reaches a correspondingly greater size before regressive changes have time to appear.

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THE PROPAGATION OF THE ROUS SARCOMA NO. 1 IN DUCKLINGS.

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HITHERTO all attempts to transfer the Rous fowl-sarcoma to ducks have failed. For example, Rous (1911) failed, Gye failed on numerous occasions (private communication), and, more recently, des Ligneris (1932) has failed. In fact most attempts to transfer this tumour to any species of animal alien to the fowl have failed. Des Ligneris (1932) reported the transference of the tumour to turkeys and guinea-fowls, but in the former species he was never able to pass beyond the first tumour-generation, and in the latter he failed to pass beyond the third. Andrewes (1932) found it possible to transfer the tumour to pheasants, but could not transmit it beyond the second generation. Turkeys, guinea-fowls and pheasants, especially the latter, are all more closely related to fowls zoologically than are ducks.

It has now been found, as will be described, that under certain restricted conditions the Rous sarcoma No. 1 can be propagated in ducks. That the tumour will grow in ducks was shown in the following experiment:

On February 9th, 1932, a fowl bearing a rapidly-growing Rous sarcoma No. 1 was killed. Its tumour presented every appearance of high activity. A portion of the tumour was minced with scissors, and used for the inoculation of two 14-day-old ducklings. One of the ducklings, No. 548, received 0·5 c.c. of the minced tissue into the muscles of one leg only, the right; the other duckling, No. 549, received 0·5 c.c. into the muscles of each leg. Tumours developed in both birds. Duckling 549 was killed on February 15th. In each leg there was a tumour 15 mm. in diameter. Each tumour, post-mortem, was found to consist of healthy, white, soft, compact tissue, in the midst of which were one or two cleft-like spaces containing fluid. There was no capsule; each tumour was adherent to the surrounding muscles of the host, and appeared to be infiltrating them. Thus these duck tumours differed considerably from the usual fowl-grown Rous tumour. In particular they were less vascular, showed no tendency to fungate, and contained little or no sticky fluid. Histologically the tumours were pure spindle-celled sarcomas rather than myxosarcomas, and presented areas of necrosis. Duckling 548, the one which had been injected in one leg only, died of its tumour on February 20th. The tumour in this duckling had grown more slowly than the tumour in duckling 549, but post-mortem presented the same appearances.

The new departure in this experiment seemed to lie (*a*) in the use of an unusually large quantity of tissue as the inoculum, and (*b*) in the use of very young ducklings. Many experiments have been carried out on these lines,

but only general impressions and conclusions derived from these experiments will be recorded, and those only briefly.

That the quality of the mince inoculated is of importance is suggested by a comparison of the tumours obtained in different experiments, but the question has not been deliberately investigated. Neither has the optimum dose of minced tissue been worked out. Usually 0·5 c.c. has been given. Sometimes 0·25 c.c. of tissue has given rise to a large tumour, but doses smaller than this have proved less effective. Doses larger than 0·5 c.c. have seldom been given. These doses, it should be observed, relate to mince which contains no air-bubbles. When, in the process of mincing, air-bubbles had become entangled in the mass—which was seldom—they were removed by means of a vacuum pump. The doses used by des Ligneris for his turkeys and guinea-fowls were of the order of 2 c.c.; in ducks he tested doses as great as 5 c.c. But des Ligneris does not seem to have used very young birds.

Whether the variety of the ducks is of importance cannot be stated. Throughout these experiments only ducks of the Khaki Campbell variety have been used.

The age of the host is very important. In 1-day ducklings 0·5 c.c. of minced tumour-tissue has always given rise to a daughter-tumour; and perhaps the same may be said of ducklings up to 4 days old. But when ducklings of 11 or 14 days have been used, the chances of producing a tumour have been reduced considerably—perhaps by a half. In adult ducks tumours have never been obtained.

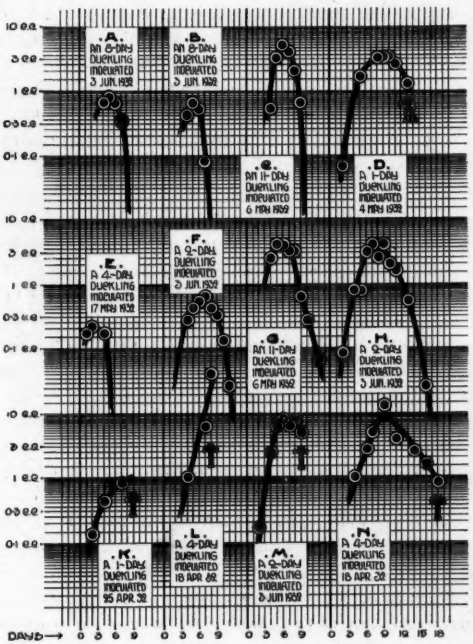
The individuality of young ducklings—that is of ducklings 1 to 4 days old—seems to have no great effect upon the result of an inoculation of minced tumour-tissue. If, for instance, a series of ducklings from a single hatching each receives a 0·5 c.c. dose of mince the resulting tumours all develop in much the same way.

One of the most striking features presented by the Rous sarcoma No. 1 when growing in ducklings is the brevity of its clinical course in contrast with the rapid growth of the tumour and the large size it attains. If 0·5 c.c. of minced tumour-tissue is injected deep into the muscles of a duckling—a 2-day duckling, for example—scarcely any immediate change can be observed by palpation; in fact only a just perceptible, soft, boggy mass can be found. Twenty-four hours later a distinct, firm, rather ill-defined swelling is palpable with ease. The nature of this swelling has not been investigated microscopically. The swelling steadily increases in size, and soon, becoming more clearly definable, is clinically an unmistakable tumour. Normally the tumour continues to grow steadily until about the sixth day; then growth ceases. Regressive changes set in, and the tumour is gone before the end of the second week. In exceptional instances the tumour follows a fulminating course, for without any slackening in rate of growth it becomes larger and larger, and the duckling is overwhelmed and is dead by the seventh or eighth day.

The presence of a tumour in the leg interferes with the free use of the limb —sometimes very considerably—but with the disappearance of the tumour the disability passes and the duckling becomes perfectly normal again. Disappearing tumours have not been studied histologically, and the processes by which the tumour is removed and the host tissues are repaired cannot be

described. Naked eye it is found that in disappearing tumours there are numerous foci of opaque, hard, necrotic tumour-tissue. For a short time after the regressive changes have become established, however, some semi-translucent tumour-tissue which is apparently still living and healthy can be found scattered through the mass.

The accompanying figure shows the growth-curve of a dozen representative duckling-grown Rous sarcomas taken from various experiments. The usual rapid growth and equally rapid decline of the tumours is well shown ; and so



also is the order of size to which these tumours grow. The chart, however, does not show the source of the mince injected—whether it was prepared from tumours grown in fowls or from tumours grown in ducklings—neither does it show the doses used. The mince given to ducklings D, E and K was made from Rous tumours which had been grown in ducklings, and in each instance the dose was 0·5 c.c. All the other ducklings referred to in the chart received mince prepared from Rous sarcomas which had been grown in fowls ; the doses, however, were not all alike. Duckling F received 0·05 c.c., ducklings A and B each received 0·1 c.c., duckling H was given 0·25 c.c., and ducklings C, G, L, M and N each received 0·5 c.c. It will be seen from the chart that in duckling F, although the dose injected was only 0·05 c.c., the resulting tumour

grew rapidly, and attained a size of 0·6 c.c. before regressive changes set in. From the growth curves of tumours such as that of duckling F it is inferred that the *rate* of growth of the new tumour is not affected materially by the size of the inoculum from which it originates; within the limits tested, size of inoculum seems to determine only the number of points at which the new tumour originates. The method of estimating tumour-sizes and the reason for using semi-logarithmic paper for the curves have already been discussed (Gye and Purdy, 1932).

From what has been said thus far it seems fair to assume that attempts to propagate the Rous sarcoma No. 1 in ducks will be the more likely to succeed the more nearly the following scheme is adhered to: To each of 10 or more 1-day ducklings give 0·5 c.c. of mince made from a very actively growing fowl-grown tumour; when tumours have developed in the ducklings, but not later than the fourth or fifth day, select the most actively growing of these new tumours, mince it, and inject 0·5 c.c. of the mince into each of 10 or more 1-day ducklings; continue in this way from one tumour-generation to another. It will be observed that young ducklings are insisted upon, and that the size of dose is large compared with the doses usually employed in this country for the propagation of tumours by means of cellular grafts. The high quality of the inoculum to be used is also stressed. To inoculate mince made from tumours which are growing poorly or even regressing would be to invite failure, and since tumours are apt to vary amongst themselves, it is important that when transplants are to be made, there should be a sufficient number of tumours available to allow of the rejection of all but the very best.

It has not been found possible to obtain quite the optimal conditions suggested, but the following experiment is considered adequate for the purpose in view, namely, to explore the possibility of propagating the Rous sarcoma No. 1 in ducks through an indefinite number of tumour generations.

Rous sarcoma No. 1 growing in fowls.—Each of two young chickens was given Rous sarcoma tissue into the breast muscles on February 15th, 1932. Chicken 294, the one in which the tumour grew most rapidly, was killed on the 26th. Post mortem its tumour consisted of highly active tissue.

First generation in ducklings.—The tumour taken from chicken 294 was minced and 0·3 c.c. was injected into the leg muscles of duckling 428, which was not more than four days old at the time. Soon a large tumour appeared at the site of injection. On the 9th the duckling had become moribund and was killed. The tumour was not encapsuled. It consisted of healthy, compact, white tissue in which were large, irregular, cleft-like spaces containing clear fluid.

Second generation in ducklings.—On March 9th duckling 447, which was then about a week old, received into its right leg 0·5 c.c. of tissue-mince prepared from the tumour carried by duckling 428. A large tumour developed. The duckling was killed on the 16th. The tumour closely resembled that of duckling 428 and need not be described further.

Third generation in ducklings.—On March 16th mince prepared from the tumour carried by duckling 447 was injected into two 1-day-old ducklings. Each bird received 0·5 c.c. into its right leg. Large tumours

developed in both ducklings. On the 23rd both tumours appeared to be still growing, and on that date one duckling, No. 106, was killed. The tumour from this duckling weighed 6·5 gm. and had replaced most of the muscular tissue of the thigh. The tumour could well be described in the terms used for the tumour of duckling 428 of the first generation, and had every appearance of being fully as active as that tumour. The second of the two ducklings died of its tumour on the 28th.

Fourth generation in ducklings.—On March 23rd two 1-day ducklings each received into the muscles of its right leg 0·5 c.c. of mince prepared from the tumour carried by duckling 106. One of the ducklings, No. 103, was killed on April 1st. Its tumour was large, but consisted of tissue which was harder and firmer than had been seen before in this series; there was, however, no necrosis. In the second duckling a tumour developed at the site of inoculation but it never reached large size; perhaps it grew to the size of a small pea, then the tumour began to disappear. By the 4th the tumour of this duckling was barely palpable and two days later could not be felt.

Fifth generation in ducklings.—On April 1st part of the tumour carried by duckling 103 was minced with scissors in the usual way. The tumour-tissue was tougher and more leathery than that of any previous tumour of the series; consequently the mincing was correspondingly less perfectly done and the mince was not so fine as usual. Each of three 1-day ducklings received into its right leg 0·5 c.c. of the tumour mince, and two chickens each received 0·1 c.c. Tumours developed in all three ducklings, but they grew rather slowly, and none became very large. On the 10th all the duckling tumours were alike in size and were about as big as a large pea. Afterwards the tumours showed differences in growth, although each eventually killed its host. The tumour of one of the ducklings continued to grow until, on the 18th, when the duckling died, the tumour measured 15 mm. × 10 mm. The second duckling died on the 19th, and on that date its tumour was not more than half as large as it had been on the 10th. The third duckling died on the 20th, and, post mortem, its tumour was found to be represented only by two nodules, neither of which was bigger than a millet-seed. Propagation through further generations in ducks was not attempted in this series.

The two inoculated chickens both developed large tumours. This shows that the duck-grown tumour had still retained its power of initiating tumours in fowls.

In the experiment here reported the Rous sarcoma was propagated in ducklings for twenty-three days, and in that time passed through five tumour generations in those birds. It has been found in this and similar experiments that the Rous tumour growing in ducklings pursues the same clinical course, no matter whether the mince injected has been derived from a fowl or from a duck. This is illustrated by growth-curves shown in the chart. The post-mortem appearances are also the same in the two cases. No metastases have been observed in ducklings.

At the end of the experiment there seemed no reason to suppose that the Rous sarcoma No. 1 cannot be carried on indefinitely in ducklings, provided

that the conditions of the experiment approximate closely to those which have already been suggested as optimal. No difficulty can be foreseen, except one which would arise if the tumour became infected with ordinary micro-organisms. It has already been related elsewhere (Gye and Purdy, 1931) how the Rous sarcoma in fowls is apt to become infected with a small organism, which reveals its presence, if at all, only in a decline in the malignancy of the tumour strain. In fowls the simplest way to get rid of the organism is to propagate for one generation by means of an extract which has been filtered. When the Rous tumour is being propagated in ducklings this simple method is impossible of application. Filtrates, whether made from tumours grown in fowls or in ducklings, never give rise to tumours in ducklings. Even extracts which have been freed from particles by centrifugation and not filtered have failed to produce a tumour in any dose we have been able to give to ducklings, young or old. Doses of 2 c.c. and even of 7 c.c. have all failed, except that on one occasion there was doubt as to whether or no a pin-point nodule was present in one duckling. When the corresponding test is made in fowls it is found that extracts of duck-grown Rous sarcomas produce tumours just as readily as do extracts of fowl-grown tumours. The complete failure to produce tumours in ducks by means of extracts of the Rous sarcoma appears to be noteworthy.

One other tumour—Begg's endothelioma, Mill Hill No. 2—has been tested in young ducklings. Minced tissue of this fowl tumour when given in doses of 0·5 c.c. certainly gives rise to growing tumours in ducklings, but the tumours are very small, and they have usually completely disappeared by about the end of the first week. Indeed the tumours have always been so small that propagation to further generations has seemed impossible and has not been attempted. Perhaps the largest tumour yet produced arose in a 1-day duckling as a result of the injection of 0·5 c.c. of minced tumour-tissue. This tumour reached its maximum size, 0·5 c.c., on the sixth day, and had disappeared entirely by the eleventh day. Centrifuged extracts of the fowl-grown endothelioma have always failed to produce tumours in ducklings.

SUMMARY AND CONCLUSIONS.

The Rous sarcoma No. 1 has been propagated in ducklings for five tumour-generations and, presumably, could be propagated indefinitely in these birds.

Very young ducklings were used. They were of the Khaki Campbell variety.

For the propagation of the tumour it was found necessary to use minced tumour-tissue as the inoculum. The dose was 0·5 c.c. given intramuscularly.

Filtrates of duckling-grown Rous sarcomas do not produce tumours in ducklings—neither do filtrates of fowl-grown Rous sarcomas.

Filtrates of duckling-grown Rous sarcomas do produce tumours in fowls, and do so just as readily as filtrates of fowl-grown sarcomas.

Thus the Rous sarcoma No. 1 growing in ducklings is exactly like a mammalian tumour growing in a mammal: within the species it is propagable by means of cell-grafts, but filtrates do not produce tumours. It is only because

filtrates of duck-grown Rous sarcomas give rise to tumours in fowls, an alien species, that a filter-passing agent can be demonstrated.

Minced tissue prepared from fowl-grown specimens of Begg's endothelioma will give rise to growing tumours in very young ducklings, but the tumours are never large and they soon disappear. Filtered extracts of the fowl-grown endothelioma never produce tumours in ducklings.

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THE EFFECT OF TUMOUR REGRESSION AND TISSUE ABSORPTION ON SOME PROPERTIES OF THE SERUM.

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THE occasional spontaneous regression of malignant growths of experimental animals shows that definite systemic factors are involved in the progress or regression of cancerous lesions, and, incidentally, provides grounds for optimism regarding the ultimate control of the disease. While rather more attention has been paid to the question of the cause of cancer development than to the cause of cancer regression, it is obvious that the study of the latter question has its merits, and might, indeed, lead to more rapid progress.

In our laboratory we have never met with spontaneous regressions of the Rous tumour, that is, cases in which the inoculum took well and showed rapid growth, but was ultimately brought to a standstill by the natural resistant agencies of the bird. On the other hand, it was observed that among birds which had been extensively bled for serum an occasional regression took place. Such regression might have been due to a decrease in the concentration of some factor in the blood as a result of bleeding, or it might have been related to the increased activity of the bone-marrow. Intra-venous injections of sodium oleate which would cause considerable hæmolysis and consequent stimulation

of the bone-marrow, without loss of blood, failed to clear up this question on account of the very low tolerance of the birds for the oleate. On the other hand, it was found that intra-tumoral injections of sodium oleate sometimes led to definite regression, and occasionally to complete disappearance of the tumour (*cf.* Webb, 1901); also, potent Rous filtrates were found to be inactivated if a neutralized 1 per cent. solution of sodium oleate was added in amounts varying from equal parts to 1 of oleate and 8 of filtrate.

It seemed reasonable to suppose that a study of properties of the serum in which variations may occur in cancer might throw further light on the factors associated with regression. Two such properties frequently referred to in the literature are the anti-tryptic and lipolytic powers of the serum; while it has been shown that the charge on the serum protein (tested according to Bendien by the composition of the sodium vanadate-acetic acid mixture required to produce flocculation) undergoes an alteration in cancer and certain other pathological conditions (Smith, Holiday and Marrack, 1931). We have therefore carried out some simple preliminary investigations of these serum properties in the case of animals bearing tumours or transplants of foreign tissue in varying stages of growth or regression.

As regards the anti-tryptic power, however, it was soon found (using the method of Fine, 1930) that the presence of a Rous sarcoma led to no definite increase in the thermo-labile anti-tryptic factor in fowl serum, and it was concluded that in the case of fowl tumours, at least, the study of the accompanying anti-tryptic properties of the serum was not a profitable line of investigation. On the other hand, interesting results were obtained when the lipolytic and precipitation reactions of fowl serum were examined. It was found that the lipolytic power of normal fowl serum (measured as described by Rona and Michaelis, 1911) is quite low. In most cases the lipolytic power of such serum was found to be about a twelfth that of cavy serum, though the individual variation was sometimes quite marked. The species of laboratory animals examined were in the following order of increasing lipolytic power: fowl, rat, rabbit, mouse, cavy. In the case of fowls bearing the Rous sarcoma no very definite alteration in serum lipolytic power appeared to be caused by the presence of the tumour; but it was found that the regression of a tumour was associated with an increase in lipolytic power, and that birds in which a tumour had been completely absorbed consequent on bleeding or intra-tumoral injection of sodium oleate (leaving the bird immune to a subsequent cell inoculation) had a serum lipolytic power of more than twice the normal value. This was not a direct effect of the oleate, as it was found that intra-muscular injection of oleate into a normal bird did not increase the lipolytic power.

The increase in lipolytic power during regression appeared to be associated with the process of tissue absorption, being possibly due in part to the enzymes liberated by the destruction of the tissue. Some evidence for this view was found by injecting liver from a White Leghorn into the breast of a Barred Rock fowl, whereupon the lipolytic power of the latter bird's serum, determined a week later, was increased by 140%. The lipolytic power of rat serum two weeks after the animal had been inoculated subcutaneously with mouse tumour tissue was increased by about 50 per cent., and four weeks later, when

necrosis and absorption were more marked, by 200 per cent. The lipolytic power of rabbit serum was increased by nearly 100 per cent. above its normal high value subsequent to an injection of Rous tumour tissue. The change of lipolytic power in this latter case was very great, and could hardly be attributed simply to the release of lipase from the Rous cells. We found that neither potent Rous filtrate nor a suspension obtained by gently centrifuging Rous tumour tissue ground in saline possessed any lipolytic power. The increase is therefore probably due to a blood reaction to the presence of surviving foreign tissue. As to dead tissue material, it was found that injection of an emulsion of acetone-dried spleen powder, free from lipase, led to no increase in the bird's serum lipolytic power.

The vanadium oxide flocculation of Bendien appears to be insufficiently specific for the clinical diagnosis of cancer, but was considered worth some investigation in relation to experimental cancer. In applying the reaction to the sera of laboratory animals, it was found necessary to use a wider range of mixtures than that described by Miller (1931) for use with human sera. The mixtures we used were numbered currently from 10 to 30, the convention being adopted that the number of a given mixture was the same as the number of parts of tenth normal sodium vanadate in two hundred parts of the mixture. The numbers of the mixtures therefore run in the direction of increasing pH. It was found that under the usual conditions normal fowl serum commenced to flocculate with mixture No. 14, rarely with No. 15 or No. 16, while that of fowls bearing either the Rous or Mill Hill sarcomas, with occasional exceptions in the case of slow-growing or partly encapsulated tumours, flocculated with No. 12. There is thus a typical displacement in the direction of increasing acidity associated with the presence of a tumour. Sera of fowls in which a tumour had been caused to regress completely, leaving the bird immune to subsequent cell inocula, and with a high serum lipolytic power, flocculated with No. 17. Some results obtained with mammalian sera are shown in Table I. The table shows that the presence of a tumour produces an effect on the precipitation point of the serum similar to that of the species difference between two sera, and the effect might therefore be ascribed to the "foreignness" of the tumour tissue. Evidence for this view was found by injecting mouse tumour 37 S into rats, whereupon the serum was found, after a fortnight, to flocculate with mixture No. 22 instead of No. 24, as previously.

TABLE I.—*Flocculation of Mammalian Sera with Vanadate-acetic Mixtures.*

Serum.	Mixture initiating flocculation.
Human (normal)	No. 27
Rat (normal)	,, 24
Mouse (normal)	,, 23
,, (with incipient tar-tumour) . .	,, 22
,, (with Tumour "63") . . .	,, 21
,, (with Tumour "37 S") . .	,, 20
Rabbit (normal)	,, 22
Cavy (normal)	,, 20

Again, the serum of a rabbit injected with Rous tumour tissue flocculated with No. 21, whereas before the injection it had flocculated with No. 22. The ratio of foreign tissue weight to body-weight was here much lower than in the case of the rats injected with mouse tumour. On the other hand, the serum of a cockerel injected subcutaneously with chicken liver flocculated with No. 16 both before and after the operation. Other factors may affect the flocculation, however, as it was found that intra-muscular injection of sodium oleate into the breast of a normal fowl shifted the flocculation point from No. 14 to No. 16.

Further experiments connected with the lipolytic and flocculation properties of sera are in progress.

EXPERIMENTAL METHODS.

Anti-tryptic Power of Serum.

The method of Fine (1931) was employed as follows: 1 c.c. of serum was added to each of two 50 c.c. Erlenmeyer flasks containing 7 c.c. of phosphate buffer at pH 8; one tube was then heated to 70° C. for half an hour to destroy antitryptic action. 2 c.c. of Cole and Onslow's pancreatic extract (diluted to 50 per cent. after neutralization to pH 8) was then added to each, and then some toluene to secure sterility. The flasks were incubated at 37°, the course of the reaction being followed by withdrawing 1 c.c. portions at intervals, adding 5 c.c. of water, and titrating by Sörensen's method. This was done by adding 10 drops of phenolphthalein (·05 per cent. in 50 per cent. alcohol), and then adding N/20 NaOH from a 1 c.c. serum pipette until a pink colour developed identical with that of a similar small flask containing 10 drops of the indicator in 6 c.c. of pH 9 buffer. The titre represents the "free acidity." 4 c.c. of 50 per cent. formalin (neutralized to pH 8) was then added and the titration continued until a colour was obtained corresponding to a mixture of 10 drops of indicator and 10 c.c. of pH 9 buffer in a similar flask. The further quantity of alkali required for this represents the "formol acidity."

Figs. 1 and 2 are typical of the results obtained in this way from normal fowl serum and the serum of a bird with a Rous sarcoma. It will be seen that the "formol" curves in each case show quite similar, slightly anti-tryptic effects lasting about 12 hours, at the end of which time recovery takes place, and digestion is more rapid in the case of the raw serum. The "free acid" curves show that the digest of heated normal serum has the highest free acidity throughout the reaction. The recovery phenomenon is sometimes attributed to the presence of a peptidase in the serum, which can only become active as the anti-tryptic effect wears off. The "formol" titres of heated and unheated serum from a fowl with a Mill Hill No. 1 sarcoma became identical after about 12 hours and there was no recovery, for after 30 hours the heated serum was showing more active digestion than the unheated. We were at a loss to account for this result even assuming the absence of peptidase from the serum.

Lipolytic Power of Serum.

This was measured after the method described by Rona and Michaelis: Several drops of tributyrin are shaken thoroughly with 100 c.c. of phosphate

buffer prepared by adding 44 c.c. of ·2 NNaOH to 50 c.c. of ·2M KH_2PO_4 and diluting to 500 c.c. The mixture is filtered, the first few c.c. being rejected. To 25 c.c. of this filtrate 1 c.c. of the serum is added. The drop-number of a given volume of the mixture is then determined at intervals with a suitable

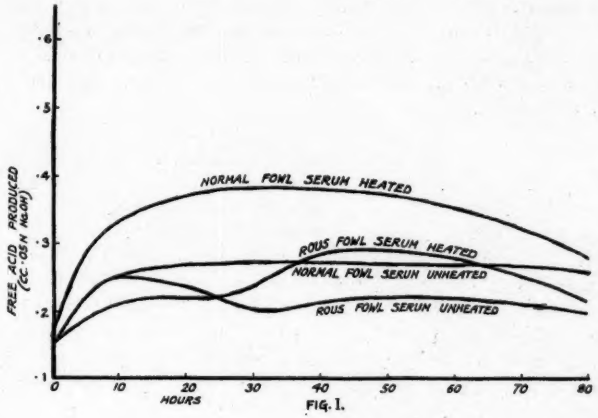

FIG. I.

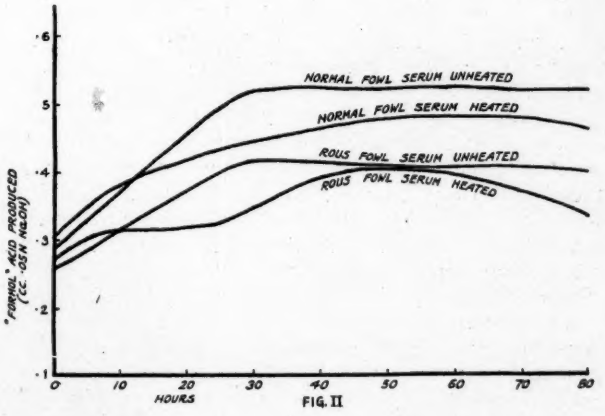

FIG. II

dropping pipette. In our case the pipette held about 3·5 c.c., was provided with a glass tap at the air-inlet and delivered 96 drops of water. The drop-number of saturated tributyrin-buffer was 160, that of buffer without tributyrin 98, so that a change in drop-number of 62 corresponded to the complete destruction of the tributyrin by the serum lipase. The curve of drop-number against concentration of tributyrin is almost linear, so that the

drop-number change is a fairly accurate measure of the percentage of tributyrin destroyed.

The tedium of drop-counting was overcome by the use of a mechanical counter, the principle of which was briefly as follows: The drops fell from a height of half an inch into the space between two vertical platinum wire electrodes, thus momentarily completing a 45-volt circuit and activating a solenoid so that it attracted a strip of soft iron attached to an escapement lever. A small horse-shoe magnet with its field almost at right angles to that of the solenoid provided the restoring force for the lever. The latter controlled a ten-toothed escapement wheel geared to a train of counting-wheels driven by a watch-spring. The escapement was designed by Mr. H. D. Purves, M.Sc., to whom we are much indebted for assistance and advice.

Preliminary measurements on fowl serum carried out at room temperature (14° C.) showed that the lipolytic power was very small, the drop-number change being 2 to 5 per hour. In order to obtain larger changes in drop number within an hour, we adopted the method of placing the reaction flask in an incubator at 37° for an hour after the first drop count, then cooling it rapidly in ice-water, and again taking the drop-count (Method 1). In this way satisfactory drop-number changes of about 8 to 20 per hour were obtained with fowl sera, and used for comparative purposes as a measure of their lipolytic power. However, this method was not suitable for comparing the lipolytic power of mammalian serum with that of fowl serum, since the higher lipolytic power of the former would lead to complete destruction of the tributyrin in less than an hour. We therefore modified our procedure by allowing the tributyrin solution to warm to 37° in the incubator, adding the serum, cooling a portion of the mixture immediately and taking its drop-number, repeating this procedure at the end of 15 minutes and so calculating the drop number change per hour (Method 2). As we found that a considerable destruction of tributyrin now occurred even before the first drop-count, as a result of the high initial velocity of the reaction, we finally adopted the method of adding the serum to cold tributyrin solution in order to determine the initial drop-count, and then adding a similar amount of the serum to another quantity of tributyrin solution at 37°, and after 30 minutes re-determining the drop number of the latter. By this method comparative measurements of the lipolytic powers of different species of sera can be obtained, if the amount of serum added is adjusted by diluting the serum, so that the reaction mixture provides much the same drop-number change per hour in all cases (Method 3). We have found it convenient to record lipolytic powers in arbitrary units, a serum being defined as having a lipolytic power of 10 units (under given conditions) if 1 c.c. of it in 25 c.c. of a saturated tributyrin solution causes a change of 10 in the drop-number of the solution in an hour, a change of 62 corresponding to complete destruction of the tributyrin.

The Vanadium Flocculation Test.

The following range of vanadate-acetic mixtures was prepared, the number of each mixture being the same as the number of parts of tenth normal sodium vanadate solution in 200 parts of the mixture.

No. 10 5 c.c. ·1 N Na_3VO_4 95 c.c. ·1 N CH_3CooH
,, 11 5·5 ,, ·1 N Na_3VO_4 94·5 ,, ·1 N CH_3CooH
,, 12 6 ,, ·1 N Na_3VO_4 94 ,, ·1 N CH_3CooH
,, 13 6·5 ,, ·1 N Na_3VO_4 93·5 ,, ·1 N CH_3CooH

etc., up to—

No. 30 15 c.c. ·1 N Na_3VO_4 85 c.c. ·1 N CH_3CooH

In order to enable tests to be carried out on the sera of small animals, a small-scale modification of the usual procedure was adopted. Quantities of 0·1 c.c. of water and 0·1 c.c. of the serum under examination are added to a numbered range of small Wassermann tubes, and the tubes well shaken. To each tube is added 1 c.c. of the appropriate vanadate-acetic mixture, the contents are mixed, and the tubes are allowed to stand for 24 hours. The tube of lowest number in which flocculation has taken place is then noted. Though Bendien applies this test to the heated as well as the unheated sera, we have up to the present confined our attention to the behaviour of unheated serum in the flocculation test.

The protocols of typical animal experiments, referred to earlier, are now given. Animals used in comparative experiments received identical diets.

Effect of Bleeding in Producing Regression.

Experiment of 24.6.31.—Hen No. 20 was inoculated with Rous cells 24.6.31 and a growing tumour resulted. Was bled from the wing vein for serum (10 to 20 c.c. of blood taken) 10.7.31. Growth quite disappeared 9.10.31. Was reinoculated together with controls 17.11.31. Resistant to cellular graft while controls "took" as usual.

Experiment of 3.9.31.—Hen 13 was inoculated with Rous cells 3.9.31 and growing tumour resulted. Bled for serum (10 to 20 c.c.) 9.10.31. Growth quite disappeared after some weeks. Was reinoculated together with controls 17.11.31. Proved resistant to cellular inoculation while controls "took" in the usual manner.

Effect of Intra-tumoral Injection of Sodium Oleate in Producing Regression.

Experiment of 5.2.32.—Cockerel No. 66 inoculated with Rous cells 5.2.32. Tumour resulted, and laboratory charts recorded rapid progressive growth. On 12.2.32 was inoculated intra-tumorally with 5 c.c. of 1% sodium oleate neutralized with hydrochloric acid. Tumour gradually receded, and had completely disappeared by 5.3.32. It was reinoculated together with controls 13.4.32. Proved resistant while controls "took." The sodium oleate used in these and subsequent experiments was purified by extraction with alcohol in a Soxhlet apparatus.

Experiment of 13.4.32.—Hen No. 107 was inoculated with Rous cells 13.4.32. Tumour resulted, and charts recorded usual rapid growth. Injected intra-tumorally 9 c.c. of 4% sodium oleate 5.5.32. Tumour gradually receded. till only a small hard residue remained 7.7.32. Was then re-tested with Rous

cells along with controls. The latter "took," while No. 107 proved resistant, and the small residue had disappeared on 25.7.32.

Temporary Regression.

Experiment of 5.1.32.—Cockerels Nos. 50 and 51 were inoculated with Rous filtrate 5.1.32. Both grew as shown by laboratory charts. No. 51 was inoculated intra-tumorally 20.1.32 with 5 c.c. of 1% sodium oleate. Regressed slowly till 4.2.32, when a few pin-heads only were palpable. Reinoculated 4.2.32 with a further 5 c.c. of 1% sodium oleate. Thereupon tumour grew rapidly and the bird died 16.2.32. No. 50 (untreated) died 1.2.32.

Experiment of 1.2.32.—Cockerel No. 58 was inoculated with Rous cells 1.2.32. The chart on 8.2.32 recorded a huge growth into which 5 c.c. of 1 per cent. sodium oleate was injected. There was marked regression, chart of 10.2.32 showing the tumour reduced to about one-quarter the original size. By 15.2.32 the growth was almost impalpable. By 23.2.32 rapid growth was again taking place and the bird died 3.3.32. No metastases were found.

Lipolytic Power and Flocculation Test of Sera of Normal, Tumour-bearing, and Cured Fowls.

Experiments of 6.6.32.—Four normal fowls had serum lipolytic powers of 8, 13, 11 and 10 units (Method 1) respectively. In the Bendien test flocculation took place with No. 14 mixture. Two fowls with Rous sarcoma had serum lipolytic powers of 16 units and 8 units and flocculation took place with No. 12. A fowl with Mill Hill No. 1 sarcoma had a serum lipolytic power of 11 units and flocculation took place with No. 12.

Experiments of 8.6.32.—Hen No. 20, which had been cured by bleeding (see protocol above), had a serum lipolytic power of 33 units (Method 1), and in the Bendien test flocculated with No. 17. There was turbidity without settling in Nos. 12 to 16.

Hen No. 13, cured by bleeding (see above), had a serum lipolytic power of 28 units and flocculated with No. 17.

Cockerel No. 64 had received several intramuscular injections of filtrate of mouse tumour 63 before being inoculated with potent Rous filtrate on 20.4.32. Tumour appeared in 17 days and grew slowly. Inoculated intra-tumorally with 10 c.c. of 1 per cent. sodium oleate 13.5.32. Regressed and disappeared in 18 days. Serum lipolytic power 8.6.32, 29 units. Flocculated with No. 17.

Effect of Injection of Sodium Oleate on the Serum Lipolytic Power and Flocculation Test of a Normal Fowl.

Experiment of 9.6.32.—Cockerel No. 118 (normal) was bled (10 c.c.), and the lipolytic power of its serum found to be 13 units (Method 1). In the Bendien test it flocculated with No. 14. It was injected intra-muscularly 9.6.32

with 5 c.c. of 2 per cent. sodium oleate. Bled next day. Lipolytic power 7 units, flocculated with No. 16, and showed a turbidity in Nos. 14 and 15. Again bled 15.6.32. Lipolytic power 6 units. Inoculated with Rous cells 17.6.32. By 27.6.32 was carrying a large growth; the serum lipolytic power was 8 units and the serum flocculated with No. 13.

Lipolytic Power and Flocculation Test of Sera of Laboratory Animals.

Experiments of 17.6.32.—Two normal rats had serum lipolytic powers of 28 and 32 units (Method 2) respectively. In the Bendien test the serum flocculated with No. 24. A normal rabbit had lipolytic power 76 and flocculated with No. 22. Pooled serum from six normal mice had lipolytic power 176 units and flocculated with No. 23.

Two cavies had serum lipolytic powers of 160 and 200 units and flocculated with No. 20.

Flocculation Test on Sera of Tumour-bearing Mice.

Experiment of 15.7.32.—Six mice, carrying large 63 tumours, were bled and the serum pooled. Flocculation took place with No. 21.

Experiment of 22.7.32.—Three mice with large 37 S tumours were bled. The pooled serum flocculated with No. 20.

Experiment of 23.7.32.—Two mice which had been tarred for 5 months at weekly intervals and were showing incipient tumours were bled. The pooled serum flocculated with No. 22.

Effect of Inoculation of Fresh Chicken Liver Emulsion on the Serum Lipolytic Power and Flocculation Test of a Normal Fowl.

Experiment of 16.6.32.—Normal cockerel No. 135 (Barred Rock) was inoculated intra-muscularly with 3 c.c. of liver emulsion from a normal chicken —a cross between Barred Rock cock and White Leghorn hen. It was bled previous to this inoculation, the lipolytic power of the serum being 25 units (Method 2) and flocculation taking place with No. 16. Seven days later it was re-bled. Lipolytic power 60 units; flocculation with No. 16.

Effect of Inoculation of an Emulsion of Fat-free Spleen Powder on Serum Lipolytic Power.

Experiment of 21.7.32.—Hen No. 151 was bled and the lipolytic power of the serum found to be 35 units (Method 3). It was inoculated with 4 c.c. of a saline emulsion containing 1 gm. of desiccated acetone-extracted pig spleen powder. An extract of this powder with dilute ammonia had proved to be devoid of lipase. The fowl was re-bled 23.7.32. The serum lipolytic power was 32 units.

Effect of Inoculation of Living Rous Cells on the Lipolytic Power and Flocculation Test of a Rabbit's Serum.

Experiment of 1.7.32.—Normal rabbit No. 7 (Chinchilla) was bled from the ear and the serum tested: lipolytic power 76 units (Method 2), flocculation

with No. 22. It was inoculated subcutaneously with 4 c.c. of an emulsion of Rous cells 1.7.32. Re-bled 6.7.32. Lipolytic power 144 units; flocculation with No. 21, slight flocculation with No. 20. Again bled 12.7.32. Lipolytic power 149 units. Flocculation with No. 22. Palpation showed only a necrotic residue of the inoculum.

Effect of Inoculation of Mouse Tumour 37 S Cells on the Lipolytic Power and Flocculation Test of Rat Serum.

Experiment of 20.6.32.—A normal rat was bled; serum lipolytic power 28 units (Method 2); flocculation with No. 24. Two normal rats then inoculated each with 1·3 c.c. of 37 S cells subcutaneously. In both rats the cells showed temporary growth and, during this phase, one was bled (4.7.32). Lipolytic power 46 units; flocculation with No. 22. On 21.7.32 the remaining rat showed almost complete absorption of the inoculum. Its serum had a lipolytic power of 92 units, and flocculated with No. 22.

SUMMARY.

(1) Extensive bleeding or intra-tumoral injections of sodium oleate into fowls sometimes leads to regression of the Rous tumour.

(2) The presence of the Rous sarcoma does not increase the anti-tryptic property of fowl serum.

(3) Fowls immune to cell inoculations of Rous sarcoma have a high serum lipolytic power, and the same applies to animals which have received intramuscular or subcutaneous injections of surviving foreign tumour tissue.

(4) The presence of Rous, Mill Hill, "63," "37 S," tar tumours, or of injected foreign tissue, alters the serum of the tumour-bearing animals so that the point of flocculation with vanadate-acetic acid is displaced in the direction of lower pH.

In conclusion, we desire to record our appreciation of the services of Mr. W. H. Hall, our laboratory technician, in connection with this work.

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THE USE OF EQUIVALENT PROPORTIONS OF ANTIGEN AND SERUM IN ABSORPTION OF PRECIPITIN.

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THE serological methods used in bacteriology have a restricted application in the study of the micro-fungi. The value of the direct agglutination reaction is affected by the existence of widespread antigenic group associations, which seem to increase in complexity with the evolution of the fungus, and, although agglutinin-absorption gives more specific results, the use of all agglutination reactions is limited by the need of suitable cell suspensions. The precipitation reaction, using a polysaccharide obtained by watery extraction of the fungus as antigen, is less catholic in its associations than the agglutination test, but, in its unrefined form, it is not a specific test. Precipitin-absorption has been used by Tomcsik (1930) and Stone and Garrod (1931). It gives a sharp differentiation between many species, but, if the test involves the use of a considerable excess of antigen, it may fail to detect the small antigenic difference between closely related species.

A technique in which the dose of absorbing heterologous antigen used is equivalent to its antibody counterpart in the serum appears to offer the most satisfactory means of segregating antigenic types, and, as a guide to the proper antigenic dose, the Dean and Webb (1926) optimal flocculation, antigen-antibody ratio, which appears to be coincident with the equivalent antigen-antibody ratio, may be used.

All of the work dealt with in the present paper refers to yeast-like fungi included in the families *Endomycetaceæ* (Schroeter), *Saccharomycetaceæ* (Reess), and *Mycotorulaceæ* (Ciferri and Redaelli). Nearly all species of these fungi yield, by watery extraction, a quantity of gummy polysaccharide material, which may vary somewhat in its physical character according to the fungus.

The Antigen.

The antigen is prepared as follows: Young cultures in a state of active growth on glucose-sugar are washed off with neutral distilled water, the growth from a dozen 3½ in. Petri plates being added to about 50 c.c. of water. The suspension is then heated to 115° C. in the autoclave for 20 minutes and, after centrifuging, the supernatant liquid, which is slightly acid in reaction, is concentrated to a convenient bulk *in vacuo* and treated with alcohol to a concentration of 75% to precipitate the gum. The precipitate, washed in 75% alcohol, dried, weighed and dissolved in isotonic saline containing 0·25% of phenol, constitutes the stock antigen. This method of preparing the antigen

was adopted after acid and alkaline extracts and slow autolysates had been tried.

The fractions precipitated by alcohol at 75 per cent. seem to include all the antigenic elements necessary, and no increase of specificity is gained by adding the fractions precipitated by alcohols up to 95 per cent. or by using the total watery extract, which would necessarily increase the quantity of impurity present. In Table I a comparison is made of the results of absorbing the homologous precipitating serum with three different antigens prepared from the same extract of *Mycotorula* "P"—the total watery extract, the 92 per cent. alcohol precipitate and the 75 per cent. alcohol precipitate. The antigen solutions were adjusted to give a uniform Dean and Webb optimal ratio of antigen 1/2 to serum 1/15, and absorption of a 1/15 dilution of the serum was done in every case with four strengths of the antigen—1/1, 1/2, 1/4 and 1/8. The supernatants from these twelve absorptions were tested with a 1/8 dilution of each antigen and a 1/10 dilution of the serum.

TABLE I.—*Supernatants after Absorption of "P" Serum by three "P" Antigens (Whole Extract, 92 per cent. Alcohol Precipitate, and 75 per cent. Alcohol Precipitate).*

"P" serum at 1/15 was absorbed by each antigen in dilutions of 1/1, 1/2, 1/4 and 1/8. (Opt. ratio "P" serum 1/15: "P" anti. 1/2.)

Supernatants were tested with a 1/8 dilution of each antigen and a 1/10 dilution of "P" serum; results below:

Supernatants from absorption by—	Antigens. Whole extract 1/8.	92 per cent. precipitate 1/8.	75 per cent. precipitate 1/8.	"P" serum 1/10.
Whole extract 1/1	—	—	—	+
,, 1/2	—	—	—	—
,, 1/4	+	+	+	—
,, 1/8	+	+	+	—
92 per cent. precipitate 1/1	—	—	—	+
Ditto 1/2	—	—	—	—
,, 1/4	+	+	+	—
,, 1/8	+	+	+	—
75 per cent. precipitate 1/1	—	—	—	+
Ditto 1/2	—	—	—	—
,, 1/4	+	+	+	—
,, 1/8	+	+	+	—

The results were identical for the three antigens, and showed that by absorption in equivalent ratio (antigen 1/2 to serum 1/15) each exhausted the antibody for all three, and that in the ratio of antigen 1/4 to serum 1/15, none exhausted the antibody affecting any of the three. Taking into account the dilution of the serum and the spacing of the absorbing doses, the experiment shows that the 75 per cent. alcohol prepipitate contained an antigen as complete as that of the whole extract.

The Precipitating Sera.

Precipitating rabbit sera were prepared by weekly intravenous injection of increasing doses of young cultures killed by heating to 60° C., followed , in some instances, by living cultures for the last few injections. The serum was collected when the titre was sufficiently high—usually from the 8th to the 12th day following the last injection. The quantity of the antibody response varied considerably, and not more than about one-third of the animals yielded satisfactory sera. Occasionally a good response was obtained after only six injections, but very often a second series of injections, after an interval, was found necessary.

The Relation of the Optimal Agglutination Ratios to the Equivalent Ratio.

Table II shows the result of a precipitation test with *Mycotorula* " M " antigen and the homologous serum, arranged to find the Dean and Webb optimal ratio and to show the relation of this ratio to the equivalent antigen-antibody ratio. The figures represent the times of flocculation in minutes, and the results should be read transversely. The position of optimal flocculation in each row is underlined, and the order of flocculation of the other tubes in the row may be followed.

TABLE II.—*Mycotorula " M " Serum and the Homologous Antigen Tested for the Dean and Webb Optimal Flocculation Ratio.*

(Time of flocculation is stated in minutes.)

Antigen "M"	1/6	1/12	1/24	1/48	1/96
Serum "M" 1/5	1½	2	4	9	18
,, 1/10	4¾	4	6	11	21
,, 1/20	21	11	10½	14	25
,, 1/40	—	60	28	24	37
,, 1/80	—	—	190	100	70

Supernatants from above Tested for Antigen Excess (AE) and Serum Excess (SE).

—	SE	SE	SE	SE
AE	—	SE	SE	SE
AE	AE	—	SE	SE
AE	AE	AE	—	SE
AE	AE	AE	AE	—

In the tests of supernatants, a — represents no reaction with serum or antigen.

This table may be read also as a series of five vertical rows in which a uniform quantity of antigen is measured with diminishing quantities of serum, as in the Ramon test, and it will be noticed that the earliest flocculation in each column occurs in the tube containing the greatest proportion of serum ; but the table does not include the true Ramon (1922) optimal ratio. After

48 hours in the cold the supernatants from the precipitation test were collected by centrifuging and every one was divided into two parts, one of which was tested with an appropriate dose of "M" serum to seek for antigen excess (AE), and the other with "M" antigen to seek for serum excess (SE). The results are shown in the lower part of the table, the positions of the tubes being as in the original test.

In every row the supernatants to the right of the Dean and Webb O.R. tube show serum excess and those to the left antigen excess, while the Dean and Webb O.R. corresponds to the equivalent antigen-antibody mixture.

Table II also shows that the time of flocculation of the Dean and Webb O.R. mixture increases in a definite ratio with dilution, but this ratio may not be the same as the ratio of dilution; in this result the time of flocculation increases nearly 2·7 times for every two-fold dilution of the antigen-antibody mixture. This *time ratio of optimal flocculation* is more clearly shown in Table III, which records the result of another experiment with "M" serum and a fresh "M" antigen. The table is self-explanatory.

TABLE III.—*Time Ratio of Flocculation of Optimal Mixture Dilutions.*

Opt. ratio, M. serum : M. antigen : : 1 : 1.

M. serum.	M. antigen.	Time.
1/5	1/5	1·50 minutes.
1/10	1/10	3·75 ,,
1/20	1/20	9·25 ,,
1/40	1/40	24·00 ,,
1/80	1/80	58·00 ,,

Incubation at 50° C. The time interval has increased approximately 2·5 times with every twofold dilution.

The value of the Ramon optimal ratio as a standard in toxin-antitoxin neutralization is not questioned, but, at least in the polysaccharide-precipitin reactions, it seems to imply an excess of antibody. Table IV gives the result of a test to find the Ramon O.R. for a *Mycotorula* "B" serum and the homologous antigen. Reading transversely, a uniform dose of antigen is applied to a series of graduated dilutions of serum, as in the Ramon test. The position of primary flocculation is indicated by a double underline, and it will be noticed that the Ramon O.R. does not become apparent until a considerable dilution of the antigen (1/320) has been reached. The position of primary flocculation in the vertical rows has been recorded also, and is shown by a single underline; in the second, third, fourth and fifth columns it corresponds to the Dean and Webb O.R. The lower part of the table, which gives the results of examination of the supernatants for serum excess (SE) and antigen excess (AE), shows that the Dean and Webb O.R. and the equivalent ratio are coincident, but the Ramon O.R. implies a considerable excess of serum; in this case eight equivalent doses for the antigen dilution, but the actual antibody residue in the Ramon O.R. supernatant has not been measured. These results are in harmony with the findings of Taylor (privately communicated) in his work with crystalline egg albumen-antigen and antiserum.

TABLE IV.—*Mycotorula "B" Serum and Antigen. Test to find Ramon opt. ratio.*

Myco. "B" serum . dils.	1/2·5	1/5	1/10	1/20	1/40	1/80	1/160	1/320
Myco. "B" antigen . 1/40	+++	+++	++	+	os	—	..	..
„ . 1/80	+++	+++	++	+	o	—	..	..
„ . 1/160	+++	++	++	±	+s	0s	..	..
„ . 1/320	++	++	++	+	±	+vs	—	—
„ . 1/640	+	+	±	+	+	+vs	0tr	—

Supernatants from Above Titrated with Appropriate Doses of Antigen and Serum.

SE	—	AE	AE	AE	AE	.. ..
SE	SE	—	AE	AE	AE	.. ..
SE	SE	SE	—	AE	AE	.. ..
SE	**SE**	SE	SE	—	AE	AE AE
SE	SE	**SE**	SE	SE	—	AE AE

+ = Precipitation (s = slight, vs = very slight, tr = trace).
o = Opalescence.
— = No change.
The position of primary flocculations in the transverse rows marked by a double underline and in the vertical rows by a single underline.
SE = Serum excess. (**SE** = Supernatant from Ramon O.R.)
AE = Antigen excess.

The Effects of Absorption by a Heterologous Antigen.

Reactions between heterologous sera and antigens yield results similar to those described above and, presumably, they depend upon the interaction of group elements; so that the absorption of a serum by an equivalent dose of a heterologous antigen should remove all the corresponding group fractions of the antibody, leaving the *specific* portion intact. Table V gives the result of a precipitation reaction between a *Mycotorula* "C" serum and the heterologous *Mycotorula* "T" antigen. The equivalent ratio had been determined previously as "C" serum 1/10 to "T" antigen 1/15, and the test is arranged so that this ratio occurs in the middle tube, with antigen excess in the two tubes on the left and serum excess in the two on the right.

TABLE V.—*Mycotorula "C" Serum and the Heterologous Mycotorula "T" Antigen. Test for Dean and Webb O.R. and Examination of Supernatants.*

Myco. "T" antigen dils.	1/2	1/2·5	1/3	1/4	1/5
Myco. "C" serum 1/2	+++	+++	+++	+++	+++
Supernatants:					
Antigen "C"	++	++	++	+++	+++
Serum "C"	++	+	—	—	—
Antigen "T"	—	—	—	+s	+
Serum "T"	++	++	+	+s	+vs

Position of the Dean and Webb O.R. is marked by an underline.

The supernatants from all five tubes were divided into four parts and tested with the homologous and heterologous antigens and sera; the results are shown in the lower part of the table. In the first row the homologous "C" antigen gave strong precipitation with all supernatants, greatest in the position of "C" serum excess and diminishing towards the left with increase of "T" antigen excess. In the second row the homologous "C" serum gave precipitation only in the presence of "T" antigen excess, and, in the third row, the heterologous "T" antigen gave relatively weak precipitation in the region of "C" serum excess; these reactions, involving only those group factors responsible for the original precipitations, show that the O.R. tube contained the group antigen-antibody factors in equivalent ratio and its supernatant is free from these elements. In the fourth row the heterologous "T" serum gave strong precipitation with the first two supernatants, which contained the excess of "T" antigen, but it also gave precipitates with the supernatants from the O.R. tube and the two tubes to the right of it. This reaction, which was not given with the homologous "C" serum, suggests the presence in the O.R. supernatant of some portion of the "T" antigen which has no counterpart in serum "C"; that is the specific part of the "T" antigen. In many antigens, capable of provoking specific antibody, a specific fraction cannot be detected in this way, and it seems possible that in such cases it may be inseparable from the remainder of the antigen, or, being a minor element, it may be completely adsorbed to the group antigen-antibody complex and removed with the precipitate.

The reactions shown in Table V may be explained by the hypothesis illustrated in Table VI: The *Mycotorula* "C" serum contains an antibody group "C" which, although probably not strictly specific for the type *Mycotorula* "C," has no counterpart in the *Mycotorula* "T" antigen, and a second antibody group "CT" which reacts with both *Mycotorula* "C" and *Mycotorula* "T" antigens. The "C" antigen contains "c" and "ct" groups corresponding to the antibody groups, and the "T" serum and "T" antigen possess respectively "T" and "TC" antibody groups and "t" and "tc" antigen groups.

TABLE VI.

Antigen "T"	5, t ct	4, t ct	3, t ct	2, t ct	t ct
Serum "C"	3, C CT	3, C CT	3, C CT	3, C CT	3, C CT
Supernatant	3, C 5, t 2, ct	3, C 4, t ct	3, C 3, t —	3, C 2, t CT	3, C t 2, CT
Antigen "C"	c ct	c ct	c ct	c ct	c ct
Serum "C"	C CT	C CT	C CT	C CT	C CT
Antigen "T"	t ct	t ct	t ct	t ct	t ct
Serum "T"	T CT	T CT	T CT	T CT	T CT

For simplicity, in Table VI, the specific and group elements in antigen or antibody are shown present in equal amount, and the antigen dilutions in the

first part of the table are expressed as 5, 4, 3, 2 and 1 units corresponding to similar units of antibody ; in the equivalent ratio there are three units of each. Assuming that the reactions shown in the first part of the table involve only group factors, without adsorption of other elements, the composition of the supernatants would be approximately as shown in the second part of the table. In the third part the elements reacting with their counterparts in the supernatants are underlined, and it will be seen that the only *specific* reactions are those between the *Mycotorula* " C " antigen and the specific group of the homologous antibody in the first three supernatants of the first row, and those between the *Mycotorula* " T " serum and the specific fraction of the *Mycotorula* " T " antigen in the O.R. supernatant and the two to its right in the last row. The last reaction, as mentioned above, is not constant.

The specific reaction between the *Mycotorula* " C " antigen and the supernatants of the first three tubes may suggest that absorption in equivalent ratio is unnecessary, and that the use of some excess of antigen may be a safeguard. It is true that when the antigenic link between species is relatively weak, absorption with a considerable excess of the heterologous antigen may not seriously impair the precipitation with the homologous antigen, but this reaction may be delayed and enfeebled or even inhibited when the species are closely related. This apparent inhibition may be due to an actual reduction of the *specific* group of the antibody by union with the excess of the closely related heterologous antigen, from which it may differ only in the grouping of the elements ; or perhaps the excess of heterologous antigen in the supernatant may be adsorbed to the new specific antigen-antibody complex which is formed on the addition of the homologous antigen, and delay or inhibit the flocculation of the complex if this is relatively small in amount. Inhibition of this kind is a common phenomenon in homologous precipitations when a considerable excess of antigen is used. Taylor (1931), referring to horse serum-antihorse serum precipitation, expresses the view that the excess of antigen is adsorbed by the antigen-antibody complex, decreasing its sensitiveness and preventing the reaction going to completion. Marrack and Smith (1931, *b*), also dealing with protein antigens, state that the union of the antigen-antibody compound with further antigen is specific ; that is, the antigen must be one related to that used in the immunization.

The Effect of Group Absorption on the Specific O.R.

If the Dean and Webb O.R. be a measure of the specific, and usually major, antibody in a serum, it would follow that purely group absorption should not greatly affect the homologous O.R. of the supernatant. In a number of tests involving distantly related species, absorption in the O.R. for the heterologous antigen did not affect the O.R. for the homologous antigen, but it lowered the serum end titre of precipitation and delayed the reaction. With increasing excess of the absorbing antigen the homologous O.R. remained unaltered, but the time of precipitation was greatly increased and the range of the reaction shortened until it occurred in only a few supernatants in the neighbourhood of the O.R. supernatant. These results suggest that an excess of group antigen may, without affecting the quantity of free specific antibody, delay and weaken precipitation. When the species concerned are more closely

related, absorption with the group antigen in its O.R. may cause an alteration in the O.R. for the specific antigen, suggesting a reduction of the specific group of the antibody, but the use of an excess of the absorbing antigen may, in some cases, not cause any further change in the specific O.R., although it will delay and weaken the precipitation. It must be stated, however, that O.R. determinations with the supernatants from absorptions by closely related species have often proved unsatisfactory.

The Influence of Temperature on Precipitation.

The velocity of precipitation increases with rising temperatures, but, at relatively high temperatures (50° C.), some re-solution of the precipitate, or perhaps even dissociation of the antigen-antibody complex, may take place, followed by very slow re-precipitation after cooling. To demonstrate the effect of high temperature on precipitation the following test was made: A mixture of *Mycotorula* " P " antigen and serum in equivalent ratio was distributed in uniform quantities into four groups of tubes—A, B, C and D. Groups A and B were placed on the water-bath at 50° C., and C and D were kept on the bench at 22° C., with occasional shaking. At the end of two hours' precipitation was apparently complete in all tubes and A and B were removed from the bath. At this stage the volume of precipitate in C and D appeared to be about twice that in A and B; this difference was due chiefly to the formation of a more compact precipitate at the higher temperature. A and C were immediately centrifuged at high speed, which greatly reduced the difference in volume of the precipitates. The supernatants from A and C were transferred to fresh tubes and all the tubes were placed in the cold. After three hours a marked opalescence had developed in supernatant A, while C remained clear; at the end of 24 hours A showed a considerable precipitate and C was still almost clear; and at the end of 48 hours the precipitate in A had increased and C showed a very light deposit. B and D after standing 24 hours in the cold were centrifuged, and 48 hours later the supernatant from B showed a very slight precipitate, but that from D remained clear. This experiment shows that at 50° C. some of the precipitate in A was re-dissolved, and that it commenced to re-precipitate from the supernatant within a few hours of cooling, and continued to separate out up to the end of about 48 hours. The supernatant from B, which was collected 24 hours after incubation, showed only a very slight precipitate during the next 48 hours, showing that the bulk of the dissolved precipitate had re-precipitated during the first 24 hours. The late and feeble precipitation in supernatant C shows that the reaction was not completed in two hours at 22° C., and the permanently clear supernatant D shows that precipitation was complete within 24 hours. In this work absorption reactions were usually incubated at 50° C. for two to three hours, and kept in the cold for 48 hours or more before collecting the supernatant; but it would seem better to avoid the higher temperatures, and to conduct all stages of the absorption, including the mixing, at a low temperature. Marrack and Smith (1931, *a*) mention that " the compound of horse serum pseudo-globulin and its antibody is slightly soluble in 0·9% NaCl solution."

Occasionally the supernatant from a precipitation in optimal ratio was found to give opalescence or even a light precipitate on addition of more of the antigen

or the serum used in the precipitation. This reaction, which was always delayed and usually did not reach precipitation, suggests the presence in the supernatant of uncombined antigen and antibody or the unprecipitated complex, and it seemed to be associated with particular sera, especially when used in very low titre or subjected to incubation at 50° C. or over. A slight excess of antigen in absorption will prevent precipitation on addition of more of the absorbing antigen to the supernatant. Marrack and Smith (1931, *b*), using azo-protein antigen and anti-serum, record a similar phenomenon, but in their experience it was of constant occurrence.

CONCLUSION.

Many of the cultures used in this work and all of those referred to specifically in this paper were propagated from single-cell isolations made by the ultra-violet, mercury vapour lamp, irradiation method of Topley, Barnard and Wilson (1921), and the general reactions of the new strain were checked with those of the parent culture before it was accepted for use.

Approximately thirty-five precipitating sera and eighty-five antigens were used in connection with this work, and the results point to the value of equivalent dosage in precipitin absorption, especially when the test is used for the detection of minor antigenic differences between closely related species.

The Dean and Webb optimal flocculation ratio may be used as a guide to the equivalent antigen-antibody ratio.

The tests were used chiefly for the detection of specific homologous antibody in the supernatants, but some of the experiments suggest that a specific fraction of the heterologous antigen may be present also.

A method frequently used was the equivalent absorption, by a heterologous antigen, of a serum which was then used to group other heterologous antigens.

The results of specific O.R. determinations with supernatants from absorption with an excess of group antigen, suggest that the group antigen remaining over in the supernatant may, without reducing the amount of free specific antibody, delay and weaken the precipitation with the specific antigen.

The homologous optimal ratio with the supernatant after heterologous absorption may be an index of the integrity of the specific antibody group.

The avoidance of high temperatures in precipitations which are to be followed by examination of the supernatants is advocated, and in all tests involving work with supernatants the need for adequate control is emphasized.

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THE RELATION OF OPTIMAL AGGLUTINATION TO THE EQUIVALENT SERUM-SUSPENSION RATIO.

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In a previous paper (1932), dealing with the identification of species of micro-fungi by the serum-precipitin absorption test, the use of an equivalent proportion of the heterologous antigen in absorption was discussed. It seems that a similar method might, with advantage, be applied to the absorption of bacterial agglutinins, especially in relation to such serological groups as the Flexner dysentery bacilli or the Brucellas, in which serological type distinctions may depend in some measure on quantitative differences of common antigenic factors. In the latter group, Wilson and Miles (1932) have already shown the importance of using a suitable absorbing dose of bacteria.

In the serum-precipitin reaction two different antigen-serum ratios giving optimal flocculation can be found : one by the Ramon (1922) method, employed in the titration of diphtheria toxin, in which falling doses of antibody are titrated against a constant dose of antigen, and the other by the Dean and Webb (1926) method, in which falling doses of antigen are titrated against a constant amount of antibody. Of these two, the Dean and Webb optimal ratio was found to coincide with the equivalent antigen-antibody ratio, and was chosen therefore as the guide to the antigenic dose for equivalent absorption.

Optimal Ratios in Agglutination.

Optimal ratios can also be determined for bacterial serum agglutination, by the Ramon and the Dean and Webb methods. Using a potent agglutinating serum in a series of graduated dilutions, commencing in a very low titre, and a very light bacterial suspension in uniform dosage, the earliest agglutination will appear, possibly, in a tube near the middle of the series, and from the position of this primary or optimal flocculation the reaction will spread out on either side, but a definite unagglutinated prozone may be apparent for a considerable time. This phenomenon has been recorded by Heuer (1922) and by da Costa Cruz (1929). Bier (1931) has shown that by raising the tonicity of the saline diluent, the position of optimal flocculation may be displaced towards the left—that is, to a tube containing a higher proportion of serum.

The ratio of serum to bacterial suspension in the primary flocculating mixture of this system, which will be referred to as the Ramon O.R., is constant for varying strengths of the bacterial suspension and the same series of serum dilutions. With increasing strengths of the suspension the velocity of the

reaction increases, but, until relatively dense suspensions are reached, there should be no difficulty in finding the position of primary flocculation.

If the arrangement of the test be reversed by using a uniform quantity of serum with a series of graduated dilutions of the bacterial suspension, as in the Dean and Webb test for optimal ratio in precipitation, a primary or optimal agglutination ratio will also be found. This ratio, the Dean and Webb O.R., is also constant for varying doses of the agglutinating serum and the same series of suspension dilutions. The differences in opacity caused by the graduated dilution of the bacterial syspension in the Dean and Webb system make comparative reading of degrees of agglutination more difficult than in the Ramon system, but this difficulty is quickly overcome by practice.

For the same reacting system the two optima do not coincide ; in fact they may differ widely, but in such cases the ratio of the two optima is constant. Usually, as in the precipitation test, the Ramon O.R. requires several times the Dean and Webb equivalent of serum for the same dose of suspension. These findings are in harmony with the results (privately communicated) of G. L. Taylor's work with crystalline-egg-albumen antigen and antiserum.

Da Costa Cruz (1929, *b*), who employed only the Ramon method, regarded the Ramon O.R. as a balanced proportion of specific agglutinin and suspension. He used an anti-Flexner dysentery serum, which gave different optimal agglutination ratios for the homologous organism and *Bact. dysenteriæ* Shiga, and found that it could be absorbed by the " Shiga " suspension without altering its optimal ratio for the homologous organism.

The Equivalent Serum-suspension Ratio.

To determine the equivalent ratio of serum to suspension, the lower limits of serum excess and suspension excess may be sought in a series of agglutinations arranged as in the Ramon method or in the Dean and Webb method, and the position of the equivalent ratio placed between them. The limit of serum excess may be sought by testing the supernatants, after agglutination, for the ultimate residue of agglutinin ; but, as is shown below, the detection, by the agglutination test, of such minute quantities of agglutinin may be very difficult or even impossible. The fixing of the lower limit of suspension excess is scarcely more satisfactory ; in this work it has been taken as that tube in which agglutination just fails to be complete.

Table I gives a comparison of the two optimal agglutination ratios and their relation to one another and to the apparent limits of serum excess and suspension excess. A salmonella type " Dublin " serum was used with a *Bact. enteritidis* (" O " antigens) suspension of density 72×10^9 organisms to 1·0 c.c., and the end-point of agglutination was given by a 1/384 dilution of the serum with an equal volume of a 1/2000 dilution of the suspension. The protocol is based on a single experiment in which the Ramon O.R. was found by taking readings transversely and the Dean and Webb O.R. by reading the vertical columns. The position of the Ramon O.R. is marked by an **R**, that of the Dean and Webb O.R. by a **D**, and the extent of incomplete agglutination by a rule extending from the right ; the extremity, corresponding to the position of the tube in which agglutination just failed to be complete, was taken as

TABLE I.—*Salmonella "Dublin" Serum and* Bact. enteriditis *Suspension.*

	Serum dilutions.									
	1/6.	1/9.	1/12.	1/18.	1/24.	1/36.	1/48.	1/72.	1/96.	1/144.
Suspension 1/32	—	D								
Supernatant			—	—	—	—	—	—	—	—
Suspension 1/64	—	—	—	D						
Supernatant				—	—	—	—	—	—	—
Suspension 1/128	—	—	—	—	—	D				
Supernatant					—	—	—	—	—	—
Suspension 1/256	—	—	R	—	—	—	—	D		
Supernatant						—	—	—	—	—
Suspension 1/512	—	—	—	—	R	—	—	—	—	D
Supernatant							—	—	—	—
Suspension 1/1024	—	—	—	—	—	—	R*	—	—	—
Supernatant								—	—	—
Suspension 1/2048	—	—	—	—	—	—	—	—	R	—
Supernatant									—	—
Suspension 1/4096	—	—	—	—	—	—	—	—	—	—
Supernatant										—

All supernatants were titrated with a 1/2000 dilution of the suspension.
R = position of Ramon O.R. **D** = position of Dean and Webb O.R.
The horizontal lines on the left indicate extent of agglutination by supernatants; on the right the range of incomplete agglutination.

* The Ramon reading for this row was not taken synchronously with the others, but was obtained from a repetition of the test with identical reagents.

the lower limit of suspension excess. The supernatants were titrated with a 1/2000 dilution of the *Bact. enteritidis* suspension, and the results are illustrated by a rule extending from the left indicating the extent of agglutination—that is, the apparent extent of serum excess. An examination of the table will show that the Ramon O.R. and the Dean and Webb O.R. are unvarying for all dilutions of the suspension or serum, and these ratios bear a constant relation to one another and to the apparent limit of suspension excess. The apparent end-points of serum excess do not correspond to a constant serum-suspension ratio in the original agglutination, but to a series of ratios in which the bacterial equivalent is reduced approximately fourfold for every twofold dilution of the serum. This is not a fortuitous result, but one which has been observed with other sera and suspensions. A line projected on the end-points of serum excess, as judged by the testing of supernatants, would, following the successive dilutions, diverge more and more widely from the position of suspension excess, as judged by the limit of complete agglutination, leaving a considerable gap in which the equivalent ratio may occur. If such a line be accepted as a true expression of the limit of serum excess, it would imply that the degree of antibody absorption for a constant serum-suspension ratio increases with dilution. However, the results of a further experiment, described below, suggest that this line does not represent the true limit of serum excess, but only the limit

detectable by visible agglutination for the concentration of bacterial suspension used in the titration of the supernatants; this concentration (1/2000) approaches the practical limit of dilution. The agglutination test used quantitatively has only a comparative value, and the visible reaction is not sufficiently delicate to detect the ultimate fractions of agglutinin present in the supernatants lying beyond the apparent limit of serum excess. If an exact estimation were possible, it seems probable that the limit of serum excess would approximate to the Dean and Webb O.R.

In the further experiment referred to above, the Dublin serum was absorbed by *Bact. enteritidis* suspension in various dilutions in the Ramon, O.R., and the supernatants were titrated for end-point of agglutination with a 1/2000 dilution of the *Bact. enteritidis* suspension. The results of these agglutinations gave a rough estimate of the *comparative* agglutinin residue of the supernatants. In the case of a 1/3 dilution of the serum absorbed by a 1/60 dilution of the suspension, the end agglutination titre was lowered from 1/768 to 1/96; a reduction of 87½%, with serum at 1/12 and suspension at 1/240, the reduction in end agglutination titre was 81¼%, and with serum at 1/48 and suspension at 1/960 the reduction in end titre was only 75%. These results suggest that dilution lessens the degree of firm antigen-antibody union.

The end-point of agglutination is given by a 1/384 dilution of the serum with an equal volume of a 1/2000 dilution of the suspension, or, in other words, 1·0 c.c. of the 1/384 dilution of the serum contains the minimal quantity, or critical dose, of agglutinin which will cause visible agglutination with 1·0 c.c. of the 1/2000 dilution of the suspension ($35{\cdot}5 \times 10^6$ organisms) in 24 hours. The 1/48 dilution of serum shown in the table will therefore contain eight times the critical dose of agglutinin in 1·0 c.c. and, from the result of the experiment above, 75% of this would be absorbed by 1·0 c.c. of the 1/960 (1/1024 in table) dilution of the suspension, leaving two critical doses of agglutinin in 2·0 c.c. of the supernatant, or one dose in 1·0 c.c., which is just sufficient to give visible agglutination in 1·0 c.c. of the 1/2000 dilution of the suspension. Any dilution of the serum in excess of 1/48 would, after absorption in the Ramon O.R., leave a subminimal dose of agglutinin in the supernatant; reference to the table will show that no agglutinin is apparent in the supernatant from the Ramon O.R. absorption above this dilution.

A similar series of absorptions was carried out in the Dean and Webb O.R., and the results indicated that even with the larger mass of bacteria absorption was not complete, but the residue of agglutinin—approximately 3·6% of the original dose in the different dilutions—was too small to permit of reasonably accurate measurement. The critical limit was reached in the supernatant from a 1/12 dilution of the serum absorbed by a 1/42 dilution of the suspension. The table shows that in the Dean and Webb O.R. agglutinin was detected in the supernatant from the 1/9 dilution of serum, but not in that from the 1/18 dilution.

It seems clear, then, that the end-points of supernatant agglutination shown in the table are only relative to the density of the suspension used in titration and, since agglutination cannot be detected with very dilute bacterial suspensions, the determination of the exact limit is beyond the scope of the agglutination test.

The table shows that the Dean and Webb O.R. of serum 1/9 to suspension 1/32 contains a slight excess of serum, which was estimated, by titration of the supernatant, to be about 3·6% of the original quantity. It follows that at least this proportion of serum excess must be present in the supernatants from all dilutions of the Dean and Webb O.R., although the quantity is too small to be detected by agglutination. This implies that the line of the Dean and Webb O.R. is slightly overlapped by the limit of serum excess, as judged by the presence of agglutinin in the supernatants, and that the Dean and Webb O.R. and the apparent limits of serum excess and suspension excess all lie in close approximation to one another.

The protocol given in this paper refers to a single experiment with " Dublin " serum and *Bact. enteritidis* suspension, but many other tests have been made with these reagents, in which the Ramon and Dean and Webb optima have been determined separately, through a more extended series of dilutions, and also the limits of serum and suspension excess. When the two separate O.R. determintations for the same reacting system, with the results of supernatant tests, were superposed, the composite figure was in agreement with the protocol in this paper. Confirmation of the results stated was furnished by similar experiments with agglutinating sera for *Bact. aertrycke*, *Bact. paratyphosum* A and a type of *Bact. dysenteriæ* Flexner, and cultures of *Bact. paratyphosum* A, *Bact. abortus equi*, Salmonella type Dublin, and four types of *Bact. dysenteriæ* Flexner.

The application of the method of proportional agglutinin absorption, based on the Dean and Webb O.R., to the segregation of serological types, awaits trial, but a very restricted study of some Flexner dysentery types by this means has proved encouraging.

SUMMARY AND CONCLUSIONS.

In serum agglutination of bacteria two different optimal agglutinating ratios of serum to suspension can be demonstrated, analogous to the Ramon optimal ratio and the Dean and Webb optimal ratio of serum precipitation reactions. Of the two, the Dean and Webb O.R. seems to approach more closely to the apparent position of the equivalent antigen-antibody ratio, and it may prove of use as a guide to the dosage in proportional absorption by heterologous suspensions.

I wish to express my indebtedness to Mr. R. Lovell for the agglutinating sera used in this work.

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A STUDY OF THE PHOSPHORUS DISTRIBUTION IN BACTERIAL CULTURES. PART III. THE PHOSPHATASE ACTIVITY OF *B. COLI* AND STAPHYLOCOCCUS.

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In Part II of this series Gordon and Cooper (1931) showed that *B. coli* is capable of synthesizing organic phosphorus compounds from inorganic sources. The recognized dependence of most bacteria on supplies of inorganic phosphorus in their nutrient media, together with the fact, established in Part I of this series by Gordon and Marshall (1930), that organic phosphorus compounds are broken down by living cultures, suggests that many bacteria may produce enzymes to maintain their supply of inorganic phosphorus. The present research was undertaken in order to determine if phosphatase enzymes are produced.

In spite of the large amount of work recorded in the literature upon glycerophosphatase present in animal and plant tissues, yeasts and moulds, we have been unable to find any reference to its presence in bacteria.

It was found that a suspension of *B. coli* or staphylococcus containing the 48-hour growth of 10 Roux bottles in 25 c.c. of water could be completely and constantly killed by shaking for 3 hours with 5 c.c. of chloroform at room temperature, and then standing at 0° C. for 15–20 hours to allow the undissolved chloroform to settle out.

Working with this suspension, positive results for the enzymic hydrolysis of glycerophosphate were obtained in the case of both *B. coli* and staphylococcus with approximately 11 days' incubation in sealed tubes. It was found possible to lessen the incubation period and increase the glycerophosphate activity by centrifuging the bacteria from the water used to wash them off the agar, and making the suspension in Sorensen's glycine buffer of pH 8·7, before killing with chloroform. This is the optimal pH for the action of the glycerophosphatase of blood (Jenner and Kay, 1932).

The analytical method employed for the estimation of inorganic phosphorus was that of Bell and Doisy (1920), as described in Part I. It was necessary to use this method of estimation in an alkaline solution in order to dissolve the protein materials which are precipitated by molybdic acid, and to minimize any hydrolysis of organic phosphorus whilst the analysis was proceeding. The addition of trichloracetic acid was omitted, as no proteins were present which were precipitated by this reagent and not dissolved by sodium carbonate. The sample for analysis was withdrawn to a centrifuge tube and spun until quite clear. One or two c.c. of the clear liquid were then taken for the estimation.

All the experiments described in this paper were tested for sterility before and after incubation, by subculture to plates, and only those with every tube sterile are included.

The method and the explanation of the results summarized in Table II can best be exemplified by describing one experiment in detail (Table I).

DESCRIPTION OF METHOD.

Ten Roux bottles, containing a thin layer of agar, were inoculated with a few c.c. of a 24-hour broth culture of the strain selected. The bottles were incubated for 48 hours, the growth rubbed up with a glass rod and washed off with normal saline, the suspension drawn off with a pipette into a centrifuge tube, and centrifuged for two hours at a fast rate. The supernatant liquid was poured away, and the deposit rubbed with a glass rod in 25 c.c. of the glycine buffer solution (autoclaved previously), transferred to a stoppered bottle, and 5 c.c. of chloroform added. This mixture was mechanically shaken for 3 hours to a uniform white emulsion. After standing overnight at 0° C., the bottle was gently rotated to cause the chloroform drops to coalesce and suspend as much of the bacterial sediment as possible above the chloroform. The aqueous layer was then transferred to a tube, and shaken for a short time to obtain an even suspension.

Four sterile test-tubes were drawn out near the top so that they could be easily sealed. Five c.c. of the suspension was pipetted into each tube. Two tubes were heated in a boiling-water bath, and the heating continued for ¾ hour after the dissolved chloroform had been driven off. After cooling, 1 c.c. of sterile distilled water was added to one tube and 1 c.c. of sterile 2½ per cent. sodium β-glycerophosphate added to the other.* Similar additions were made to the two unheated tubes. After shaking up, 2½ c.c. were withdrawn from each tube to centrifuge tubes and spun for analysis, as previously described. The tubes were finally subcultured and sealed by drawing off the heads of the test-tubes. The pH of the unused liquid was taken before and after heating.

After incubating for 4 days (in this experiment), the necks of the tubes were broken off and the contents again subcultured—transferred to centrifuge tubes, spun till clear and the supernatant liquid analysed for inorganic

TABLE I.—*Typical Experiment.*

B. coli (strain A).		Before incubation.	After incubation.	Increase.
Water suspension .	Unheated tube .	(1) 0·069 .	(5) 0·267 .	(*a*) 0·198
	Heated tube .	(2) 0·055 .	(6) 0·079 .	(*b*) 0·024
Glycerophosphate . suspension	Unheated tube .	(3) 0·071 .	(7) 0·360 .	(*c*) 0·289
	Heated tube .	(4) 0·055 .	(8) 0·079 .	(*d*) 0·024

All figures represent mgm. of phosphorus per c.c.

The inorganic phosphorus in this solution had been determined, and a correction of 0·004 mgm. per c.c. subtracted from all readings of solutions containing glycerophosphate.

TABLE II.—*Summary of Results.*

Bacterial strain.		Experimental conditions.	Precipitation by heat.	Non-enzymic phosphorus increases.		Enzymic phosphorus increases.	
				Diffusion.	Non-enzymic hydrolysis of glycerophosphate.	Autolysis.	Enzymic hydrolysis of glycerophosphate.
Staphylococcus aureus.	A.	Buffered solutions	0·014	0·069	0·012	0·020	0·147
		4 days' incubation	..	(27)	(5)	(8)	(60)
	B.	Buffered solutions	0·005	0·014	—0·002	0·014	0·086
		3 days' incubation	..	(12)	(0)	(12)	(75)
	C.	Aqueous solutions	0·016	0·043	0·045	0·004	0·054
		11 days' incubation	..	(29)	(31)	(3)	(37)
Bacillus coli.	X.	Buffered solutions	0·017	0·058	0·028	0·332	0·102
		6 days' incubation	..	(11)	(5)	(64)	(20)
	Y.	Buffered solutions	0·040	0·173	0·009	0·175	0·155
		6 days' incubation	..	(34)	(2)	(34)	(30)
	A.	Buffered solutions	0·015	0·024	0·000	0·174	0·091
		4 days' incubation	..	(8)	(0)	(60)	(32)
	C.	Aqueous solutions	0·014	0·032	0·004	0·072	0·025
		11 days' incubation	..	(24)	(3)	(54)	(19)
Limits per cent.		Staphylococcus / *B. coli*	..	8–34	0–5*	3–12 / 34–64	37–75 / 19–32

All figures represent mgm. of phosphorus per c.c. those, in parentheses being percentages.

* Excluding Staphylococcus C., which, being unbuffered, acquired considerable acidity during incubation.

phosphorus. The results are given in Table I. The pH of the sediment and liquid remaining in the centrifuge tubes was taken approximately by the indicator method.

The increase in inorganic phosphorus which takes place in a mixture of dead bacteria and glycerophosphate solution, on incubation, may be divided into two parts. First, the increase which is due to the action of *enzymes* on the organic phosphorus compounds present—(1) glycerophosphate, and (2) the phosphorus compounds of the bacteria, *i. e.* glycerophosphatase action and *autolysis*. Secondly, an increase due to *non-enzymic* action: (1) the acid hydrolysis of glycerophosphate, and (2) the liberation of inorganic phosphorus compounds which were occluded or weakly combined with the bacterial protoplasm—which in this paper we shall call *diffusion*.

When a bacterial suspension is heated, proteins are coagulated and remove a small quantity of inorganic phosphorus from solution—this decrease we have termed *precipitation*.

The *precipitation* by heat is seen to be (Table I), (1)–(2) = 0·014, (3)–(4) = ·0016, average 0·015 (see column 1 of Table II).

The *diffusion* is given by (*b*) = 0·024 (column 2 of Table II). If any non-enzymic hydrolysis of glycerophosphate had occurred, (*d*) would be a greater figure than (*b*) and the difference would give the amount (column 3 of Table II).

The *autolysis* is given by (*a*)–(*b*) = 0·174 (column 4 of Table II).

The enzymic hydrolysis in presence of glycerophosphate is similarly given by (*c*)–(*d*) = 0·265, and this is made up of *autolysis* (0·174 as above determined) and *glycerophosphatase* action 0·091 (column 5 of Table II). In Table II all these figures are also expressed as a percentage of the total change occurring in each experiment as a result of both enzymic and non-enzymic hydrolysis —in this experiment given by figure (*c*), Table I.

INTERPRETATION OF EXPERIMENTS.

Effect of Heat.

It is necessary to consider what other effects may have occurred in the heated tube besides the destruction of enzymes—autolytic and glycerophosphatase. A considerable coagulation of protein matter occurs, and this in all probability is the cause of the precipitation of inorganic phosphorus. The solution which has been heated is slightly more alkaline than the unheated. This difference is negligible in the case of the buffered solutions, but in the case of the unbuffered experiments might increase the diffusion of phosphoric acid compounds into the liquid in the heated tubes during the subsequent incubation. This would cause the figures for autolysis and glycerophosphate action to be somewhat too low. *B. coli* Y gave an unusually bulky precipitate on heating, the effect of which is reflected in the figure for precipitation.

Non-enzymic Hydrolysis.

With coagulated proteins and dead bacteria in suspension, it is not surprising that inorganic phosphorus compounds are liberated into the medium during the incubation. The fact that this increase in inorganic phosphorus was equal

or only slightly more (0–5 per cent. difference) in the heated tubes containing glycerophosphate than in those without, shows that the hydrolysis of glycerophosphate which occurred spontaneously was very small under the conditions of the experiment. Indeed, even this difference may be the result of the solution of precipitated phosphates by the glycerophosphate (Mellon, etc., 1921) and not any actual hydrolysis. The only exception to these remarks was the experiment with staphylococcus C, which, being unbuffered, acquired some acidity during the incubation, and probably did result in slow acid hydrolysis of the glycerophosphate. In the case of the corresponding *B. coli* C experiment, the solution after incubation was still on the alkaline side of neutrality.

The question remains whether similar changes occur in the non-heated tubes. It seems reasonable to suppose that, in the absence of coagulated proteins with their occluded phosphorus compounds, the diffusion of inorganic phosphorus into the medium during incubation will certainly not be more than occurred in the heated tubes, but may be less. The values given for the enzymic hydrolysis (autolytic and glycerophosphatase), and calculated on the assumption of the equality of diffusion in the heated and unheated tubes, will thus, if anything, be lower than the true values, and may thus be regarded as expressions of the minimum amount of enzymic activity occurring.

Enzymic Activity in the Absence of Glycerophosphate (Autolysis).

The outstanding results obtained are the low autolysis figures obtained for staphylococcus (3–12 per cent.), compared with the high results for *B. coli* (34–64%). This difference may be due to (*a*) the presence of different enzymes in the two series, or (*b*) to the different nature of the organic phosphorus compounds present in the bacteria, or (*c*) the differing nature of the cell-walls as regards permeability to the compounds present, or their resistance to rupture by osmotic forces.

Kay (1928) has shown that the glycerophosphatase content of a large number of mammalian tissues is parallel to the hexose diphosphatase and nucleotidase content, and suggests that these activities are the result of one and the same enzyme. If such a generalization is true for the phosphatase enzymes of bacteria, it would appear probable that the differing ratio of autolysis to glycerophosphatase in *B. coli* and staphylococcus was not due to the production of different enzymes, but to the different chemical constitution of the two organisms.

Enzymic Activity in Presence of Glycerophosphate.

After making allowance for all the preceding methods by which inorganic phosphorus may increase during incubation, there remains a definite increase, which can only be attributed to the enzymic decomposition of glycerophosphate. The glycerophosphatase activity of the two types of bacteria investigated cannot be accurately compared without a knowledge of the quantities involved in each experiment, but if the percentage of the glycerophosphate hydrolysed in 3–6 days in the buffered solutions be calculated, figures from 12–25 per cent. are obtained for both staphylococcus and *B. coli*. In so far as these figures are significant of the activity of the phosphatase present, no difference between

the two organisms is apparent—but the comparison is complicated by the fact, established by Martland and Robison (1927) and confirmed by Roche (1931), that the activity of glycerophosphatase is greatly diminished by the presence of inorganic phosphate. The slower hydrolysis obtained in our experiments than in those of Kay (1930) with blood-plasma, may possibly be connected with the relatively large amounts of inorganic phosphorus present in the bacterial suspensions. This assumption is paralleled by the slow hydrolysis of glycerophosphate by the crude extract of "takaphosphatase" used by Akamatsu (1923), which only attained completion after some 15 days.

CONCLUSION.

We may conclude, then, that when a suspension of killed bacteria, *B. coli* or staphylococcus, is incubated with a solution of sodium β-glycerophosphate, an increase in the inorganic phosphorus content of the liquid is produced as a result of (i) the enzymic hydrolysis of the glycerophosphate, (ii) the enzymic hydrolysis of organic phosphorus compounds present in the bacteria, (iii) the non-enzymic hydrolysis of phosphorus compounds, and liberation of inorganic phosphorus from the bacterial protoplasm into solution.

The magnitude of the second cause of increase (which we have termed autolysis) appears to be greater than the first (glycerophosphatase action) in the case of *B. coli*, and less in the case of staphylococcus, but in both the presence of glycerophosphatase is definitely established. The third cause varies in magnitude for each strain chosen, and may assume considerable importance in accounting for the amount of hydrolysis obtained. Finally, it may be concluded that at pH 8·0–8·7 the non-enzymic hydrolysis of glycerophosphate (less than 5 per cent.) is negligible.

SUMMARY.

(1) The presence of glycerophosphatase in *B. coli* and staphylococcus has been established.

(2) The determination of glycerophosphatase in these bacteria is complicated by the enzymic and non-enzymic hydrolysis of organic phosphorus compounds of the bacterial protoplasm.

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